CHARACTERIZATION OF ANGPTL3 DEFICIENCY AND MOLECULAR MECHANISMS OF ANGPTL3 DEFICIENCY-INDUCED HYPOLIPIDEMIA

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Cover image: “Triglyceride content of each lipoprotein type in the blood”. Illustrated by Alexandra Robciuc

ABSTRACT

Introduction and aims: ANGPTL3 is a hepatokine and a regulator of lipid metabolism primarily via its inhibitory effect on lipoprotein lipase (LPL), an enzyme responsible for hydrolysing triglycerides (TGs) in lipoproteins. Genetic loss-of-function (LOF) mutations in *ANGPTL3* cause a distinct phenotype in humans: low levels of triglycerides, total cholesterol (TC), low density lipoprotein (LDL) and high density lipoprotein (HDL) levels in the circulation (familial combined hypolipidemia, FHBL2). In the first study, *ANGPTL3* was silenced in human hepatocytes to investigate intracellular mechanisms of ANGPTL3 deficiency. The study was repeated in human lipoprotein-producing enterocytes. In the second study, carriers of *ANGPTL3* LOF s17* were challenged with a high-fat meal to clinically characterize the effects of ANGPTL3 deficiency on postprandial lipid metabolism. In the third study, data from two large health surveys were utilized to investigate whether sequence variants in ANGPTL3 contribute to dyslipidemias in Finns and if ANGPTL3 serum levels correlate with lipid metabolic biomarkers in hypolipidemic or hyperlipidemic subjects.

Methods and results: ANGPTL3 was silenced in human hepatocytes and enterocytes using lentiviral shRNA vector. Substrate uptake, their conversion into TG, and TG/apoB secretion was measured in cells and cell culture media by enzymatic assays, ELISA assays, and labeled fatty acid and glucose substrates. As a result, we demonstrated that ANGPTL3 expression and secretion are regulated by insulin, LXR agonists, and PPARγ agonist rosiglitazone and that in hepatocytes, silencing affects the lipidation of lipoprotein particles upon insulin stimulation and increases glucose uptake in these cells. Changes in lipoprotein secretion were not seen in human enterocytes. Silencing of *ANGPTL3* decreased *CD36* fatty acid transporter expression in both cell lines and phospholipid transfer protein *PLTP* expression in hepatocytes.

S17* *ANGPTL3* LOF carriers and control subjects were challenged with a high-fat meal followed by blood withdrawal during fasting and at 2, 4, and 6 hours after a meal. Plasma lipids, lipoproteins, and key regulatory proteins were measured in the plasma samples by enzymatic assays and ELISA assays. As a result, TG values in homozygous carriers remained low throughout, whereas heterozygotes and control subjects showed a typical elevated postprandial response in TG levels.

Homozygous *ANGPTL3* LOF carriers had lower fasting non-esterified fatty acid (NEFA) values, which may result in decreased supply of FA in the liver. In the Finnish population study, one rare *ANGPTL3* variant and one carrier with abnormally high TC and LDL-C levels was identified. ANGPTL3 protein levels did not correlate with lipids or lipoprotein levels in hypolipidemic or hyperlipidemic subjects, but ANGPTL3 did positively correlate with PLTP and CETP activities, and age.

Conclusions: The cell culture experiments with *ANGPTL3*-silenced hepatocytes indicate that ANGPTL3 not only affects the clearance of lipoprotein particles in the bloodstream via accelerated activity of LPL, but may also alter hepatic glucose and fatty acid substrate utilization, VLDL lipidation, and *PLTP* expression. Homozygous *ANGPTL3* LOF carriers manifest hypolipidemia in postprandial phase, which can be considered anti-atherogenic and protective against the development of atherosclerotic cardiovascular disease. Based on the genetic data, genetic variants in *ANGPTL3* are rare and do not contribute to dyslipidemia on a population level. ANGPTL3 protein levels in hyperlipidemic or hypolipidemic subjects had no predictive biomarker value for plasma lipid levels.

TIIVISTELMÄ

Johdanto: Väitöstyön tarkoituksena oli tutkia ANGPTL3 puutoksen aiheuttamia muutoksia ihmisessä rasva-aineenvaihdunnan osalta kliinisesti, populaatiotasolla sekä solutasolla. ANGPTL3 proteiinin on osoitettu säätelevän lipoproteiinilipaasin (LPL) toimintaa ja siten osallistuvan maksasta sekä ohutsuolesta syntetisoitujen kolmiarvoisten (triglyseridi, TG) rasvojen poistoon verenkierrosta. ANGPTL3 toiminnan estymiseen vaikuttavat mutaatiot (LOF; loss of function) aiheuttavat familiaaliseen hypolipidemian fenotyypin (FHBL2), jonka pääpiirteenä on matalat veren VLDL, LDL ja HDL lipoproteiinien tasot.

Päämenetelmät: Solumalleilla tutkittiin ANGPTL3:n kudosspesifistä toimintamekanismia hiljentämällä *ANGPTL3* shRNA-lentivirus vektorilla hepatosyyteissä sekä enterosyyteissä. Tutkittavien lipidien ja proteiinien tasoja mitattiin sekä soluista että solumediasta entsymaattisilla menetelmillä sekä ELISA menetelmillä. Leimattujen substraattien avulla selvitettiin solujen metabolian muutoksia. Geenien ilmentymistä soluissa tutkittiin kvantitatiivisella PCR (qPCR)-analyysillä.

Koe-henkilöt, joihin kuului *ANGPTL3* LOF kantajia sekä kontrollihenkilöitä nauttivat rasvapitoisen aterian, jonka jälkeen kerätyistä plasmanäytteistä mitattiin lipoproteiinien, rasva-aineenvaihdunnan markkereiden sekä TG:ien, kolesterolin ja fosfolipidien pitoisuuksia.

Populaatiotutkimuksessa suomalaisten *ANGPTL3* varianttien kantajien lipiditasot määritettiin tarkoituksena selvittää *ANGPTL3* varianttien osuutta dyslipidemioissa. Verenkierrossa olevan ANGPTL3-proteiinin konsentraatio mitattiin henkilöiltä, joiden lipidiarvot vastasivat hyper- tai hypolipidemiaa, jonka jälkeen tasoja verrattiin lipidiarvoihin sekä muihin lipidimetabolian biomarkkereihin mahdollisten korrelaatioiden löytämiseksi.

Johtopäätökset: Tulosten perusteella *ANGPTL3*:n ilmentymistä soluissa säätelevät insuliini, PPARγ-agonisti rosiglitazone sekä LXR-agonistit. ANGPTL3-hiljennys aiheuttaa VLDL-partikkelien lipidaation laskua hepatosyyteissä insuliini stimuloinnin aikana sekä *CD36*- ja *PLTP*-geenien ilmentymistason laskua soluissa.

ANGPLT3-LOF kantajien kliinisessä tutkimuksessa havaittiin, että ANGPTL3-puutoksen omaavilla homotsygooteilla LOF kantajilla ei esiintynyt postprandiaalista hyperlipidemiaa, toisin kuin heterotsygooteilla kantajilla ja kontrollihenkilöillä, ja tämän fenotyypin voidaan katsoa olevan ateroskleroosia ehkäisevä tekijä. ANGPTL3 puutos aiheuttaa myös veren rasvahappojen määrän vähenemistä verenkierrossa ja siten mahdollisesti vähentää maksaan päätyvien rasvahappojen määrää ja niiden oksidaatiota.

Populaatiotutkimuksessa *ANGPTL3*-varianttien kantajien osuus oli vähäinen, ja dyslipidemiaa oli havaittavissa yhdellä haitallisen variantin kantajalla. Varianttia ei vähäisen kantajien määrän takia pystytä luotettavasti kausaalisesti yhdistämään fenotyyppien aiheuttajaksi.

ANGPTL3:n plasma tasot eivät eronneet hypo- tai hyperlipideemisten henkilöiden välillä, ja täten voidaan todeta, että ANGPTL3:n plasmapitoisuudet eivät toimi hyvänä biomarkkerina ennustettaessa veren lipiditasoja.

TABLE OF CONTENTS

ABBREVIATIONS

ABCA1: ATP Binding Cassette Subfamily A Member 1

ANGPTL: Angiopoietin-like protein

ATGL: Adipose triglyceride lipase

BSA: Bovine serum albumin

CETP: Cholesteryl ester transfer protein

CM: Chylomicron

EL: Endothelial lipase

FBS: Fetal bovine serum

FHBL2: Familial combined hypolipidemia

FPLC: Fast performance liquid chromatography

HDL: High-density lipoprotein

HDL-C: HDL-cholesterol

HL: Hepatic lipase

HSL: Hormone-sensitive lipase

IRS-1: Insulin receptor substrate 1

IHH: Human immortalized hepatocyte

LCAT: Lecithin cholesterol acyltransferase

LDL: Low density lipoprotein

LDL-C: LDL-cholesterol

LDL-R: LDL-receptor

LOF: Loss of function

LPL: Lipoprotein lipase

MGL: Monoacylgycerol lipase

MTP: Microsomal triglyceride transfer protein

PL: Phospholipid

PLTP: Phospholipid transfer protein

SRBI: Scavenger receptor class B type I

TC: Total cholesterol

TG: Triglyceride

TRL: Triglyceride-rich lipoprotein

VLDL: Very-low-density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals

I. Tikka A, Soronen J, Laurila PP, Metso J, Ehnholm C, Jauhiainen M. Silencing of ANGPTL3 (angiopoietin-like protein 3) in human hepatocytes results in decreased expression of gluconeogenic genes and reduced triacylglycerol-rich VLDL secretion upon insulin stimulation. Biosci Rep. 2014 Dec 12;34(6).

II. Minicocci I*, Tikka A*, Poggiogalle E, Metso J, Montali A, Ceci F, Labbadia G, Fontana M, Di Costanzo A, Maranghi M, Rosano A, Ehnholm C, Donini LM, Jauhiainen M, Arca M. Effects of angiopoietin-like protein 3 deficiency on postprandial lipid and lipoprotein metabolism. J Lipid Res. 2016 Jun;57(6):1097-107.

* Equal contribution

III. Tikka A, Metso J, Jauhiainen M. ANGPTL3 serum concentration and rare genetic variants in Finnish population. Scand J Clin Lab Invest. 2017 Oct 3:1-9.

None of the publications have been used in other dissertations.

In addition, some unpublished material is presented.

1 INTRODUCTION

Saturated fats, or more specifically saturated triglycerides (TGs), and cholesterol (TC) levels in the blood are major risk factors for atherosclerosis, the most common cause of cardiovascular disease. Risk factors in dyslipidemia vary, and are often multifactorial, including environmental factors, such as diet and lifestyle, and genetic factors (1). Disturbances in lipid and glucose metabolism cause obesity (2), non-alcoholic fatty liver (3), metabolic syndrome, and type 2 diabetes mellitus (4), all of which increase the risk of cardiovascular diseases.

TGs are stored in the white adipose tissue and are an important source of energy. TGs are also needed as solvents for fat-soluble vitamins, to maintain body temperature and immune defense, and to maintain healthy skin and hair. Cholesterol is a vital component of cell membranes, steroid synthesis, and bile acid formation (1).

Because triglycerides and cholesterol are not soluble in water, they are transported in the circulation in protein-coated particles called lipoproteins. Lipoproteins enter the bloodstream by exogenous and endogenous pathways. In the exogenous pathways, lipids are obtained from the diet and packed into chylomicrons (CMs) by the small intestine. In the endogenous pathway, the liver produces and secretes very low density lipoproteins (VLDL) (5). In addition, high density lipoproteins (HDLs) are secreted from hepatocytes and enterocytes.

When the amount of TG-rich lipoproteins rises in the blood (after a meal), TG-enriched lipoproteins and their lipid content are cleared from the circulation efficiently, a process facilitated by the activity of lipoprotein lipase, fatty acid transporters, and lipoprotein receptors.

Hyperlipidemia is a major risk factor in lipid accumulation within arteries and in development of atherosclerosis and atherosclerotic cardiovascular disease (1). ANGPTL3 is a regulator of fat metabolism, as it inhibits lipoprotein lipase (LPL) (6), an enzyme responsible for TG hydrolysis in CM and VLDL lipoproteins. ANGPTL3 deficiency, due to loss-of-function (LOF) mutations in the gene, causes a distinctive

phenotype in humans characterized by low levels of all lipoprotein types (CM, VLDL, LDL, HDL), and consequently, low total TG, cholesterol, apoB and apoA-I levels (7-8). Therefore, ANGPTL3 is a potential therapeutic target to lower plasma TG and TC in hyperlipidemic patients with a high risk for cardiovascular disease.

The purpose of this thesis is to elucidate the function of ANGPTL3 in lipoprotein-producing cells (hepatocytes and enterocytes) and the clinical phenotype of genetic *ANGPTL3* variant carriers. On a population level, the aim is to establish whether genetic variants in *ANGPTL3* contribute to dyslipidemia and whether ANGPTL3 serum levels predict lipid or lipoprotein levels in the general population.

2 REVIEW OF THE LITERATURE

2.1 LIPOPROTEIN METABOLISM

2.1.1 Triglycerides

Triglycerides (TGs) consist of a glycerol backbone and three fatty acid chains. Depending on the properties of the fatty acids attached, TGs can be either saturated or unsaturated. Fatty acids with only single bonds between carbons are called saturated, whereas the structure of unsaturated fatty acids includes one (monounsaturated) or more (polyunsaturated) double bonds between carbons. Unsaturated fats have lower melting temperatures and remain in liquid form at room temperature, and hence, are called oils, while saturated fats have higher melting temperatures, consequently remaining in solid form at room temperature (9).

To distinguish between different types of solid fats and oils, Figure 1 shows the chemical structures of three triglycerides. Tristearin (**Figure 1A**), the primary fat in beef, consists of three saturated stearic acids. Triglycerides in butter fat (**Figure 1B**) consist of approximately 70% saturated and 30% unsaturated fat components. Olive oil comprises mainly monounsaturated oleic acid. Sunflower oil is polyunsaturated, as it contains mostly linoleic acid (omega 6) and oleic acid (**Figure 1C**) (9).

A B C

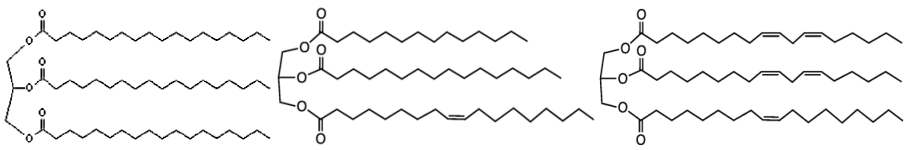

Figure 1. Types of triglycerides. A) Tristearin (3 stearic acids), B) ”butterfat (myristic, palmitic, and oleic acid), and C) sunflower oil (2 linoleic acid omega-6 and oleic acid).

An average daily calorie intake is made up of approximately 20-30% fats, or 50-90 grams, of which ideally less than 10% is saturated (10). Triglycerides in our diet are mostly stored in white adipose tissue (WAT). An individual with a normal body mass index (BMI) has a body fat percentage of 25-30% (females) or 18-25% (males) (11), which in an 80-kg male equals 16 kg of fat and 144 000 kcal of restored energy in the body, as 1 g of fat equals 9 kcal (12). By consuming an average of 2500 kcal daily, an 80-kg male can survive for 57 days in starvation by consuming his fat reservoir. Other sources of energy, protein and carbohydrates, can store only half of the energy of fats, making triglycerides the superior form of stored energy substrates (12).

2.1.2 Cholesterol

Cholesterol is a sterol, a type of lipid molecule, and a waxy substance, which comprises four linked hydrocarbon rings forming a bulky steroid structure. A hydrocarbon tail is linked to one end of the steroid and a hydroxyl group to the other end (**Figure 2 A**) (9).

Cholesterol molecules interact with phospholipids in eukaryotic cell membranes and decrease fluidity of the membrane (**Figure 2 C**) (9). The human body cannot break down cholesterol and the only disposal route for them is through the liver-gut axis (1). Dietary intake of cholesterol accounts for approximately 0.3 g daily, which is balanced by a daily loss of 0.8-1 g of cholesterol in faeces. On average, a human body contains approximately 35 g of cholesterol in total, which is either located within the cell membranes or recycled between the gut and the liver in bile. Circulatory cholesterol level in lipoproteins is approximately 1.5-2 g/l in the blood and is maintained by regulating *de novo* synthesis and dietary intake (1).

Besides cholesterol, eukaryotic cell membranes consist of phospholipid units. A phospholipid comprises a glycerol backbone attached to two hydrophobic fatty acid chains and a hydrophilic phosphate group (**Figure 2 B**). Phospholipids form a bilayer structure in aqueous solutions and together with cholesterol and proteins, constitute eukaryotic double layer cell membranes and the monolayer surface of lipoprotein particles (**Figure 2 C**)(9).

A B C

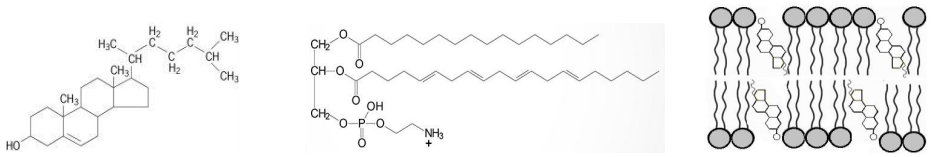

Figure 2. Lipid structures and a double-layer cell membrane. Cholesterol (A) and phospholipid (B), a eukaryotic cell membrane with phospholipid and cholesterol components (C).

2.1.3 Lipoprotein assembly

The small intestine

Triglyceride aggregates consumed in our diet enter the small intestine and are emulsified by bile and further hydrolysed into fatty acids and monoglycerides by pancreatic lipase. The breakdown of triglycerides allows the molecule to enter the enterocyte cell by simple diffusion across the plasma membrane or via specific fatty acid transporter proteins (13).

Cholesterol enters the intestinal cells via a separate mechanism, a facilitated transport by Niemann-Pick C1-Like 1 (NPC1L1) transmembrane protein. The NPC1L1-mediated uptake of cholesterol by endocytosis does not discriminate between cholesterol and structurally similar sterols derived from plants. This is why plant sterols, which cannot be metabolized further, are expelled back into the gut, through ATP-binding cassette transporter (ABCG5 and G8) -mediated active transport (13). Enterocytes have “sterol-sensors”,

nuclear transcription factors called Liver X Receptors (LXRs), which monitor sterol levels and activate the genes that regulate NPC1L1 and ABCG5/G8 (14).

Cholesterol in cells is either non-modified or converted into cholesteryl esters (CE) by acyl-CoA cholesterol acyltransferase (ACAT), which covalently attaches a fatty acid to the free hydroxyl group on the cholesterol molecule. Enterocytes pack TG and CE in chylomicron (CM) lipoproteins, which are secreted in lymph (**Figures 3 and 4**) (13).

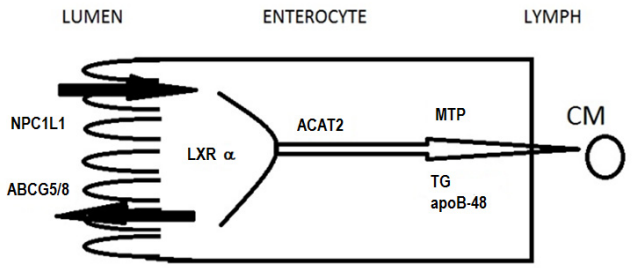

Figure 3. Cholesterol ingestion. Cholesterol uptake in the enterocyte is facilitated by the NPC1L1 transmembrane protein. Acyl-CoA cholesterol acyltransferase 2 (ACAT2) converts cholesterol into cholesterol esters, which together with TG are packed in chylomicron (CM) particles and secreted into the bloodstream.

The liver

Liver synthesizes TG from fatty acids, which originate from *de novo* lipogenesis or are taken up by the CD36 fatty acid transporter from plasma. Plasma non-esterified fatty acids (NEFAs) originate from the activated lipolysis in the adipose tissue during fasting and by the LPL-mediated lipolysis of TG-enriched CM and VLDL particles (**Figure 4**) (15).

Cholesterol is either produced *de novo* by the liver or is obtained from hepatic lipoproteins and lipoprotein remnant uptake. A large quantity of cholesterol is secreted in bile, from which it is recycled back to the liver in CM remnants (13). TG and cholesterol are secreted by the liver in very low density lipoproteins (VLDLs) (**Figure 5).** The assembly of lipoprotein particles in the enterocytes and hepatocytes both require the involvement of microsomal triglyceride transfer protein (MTP). The formation of a lipoprotein is suggested to entail co-translational lipidation of apoB with phospholipids by MTP to form a premature lipoprotein in the

endoplasmic reticulum (ER), which is then further lipidated by larger TG lipid droplets to form a mature particle in the Golgi apparatus (15, 16).

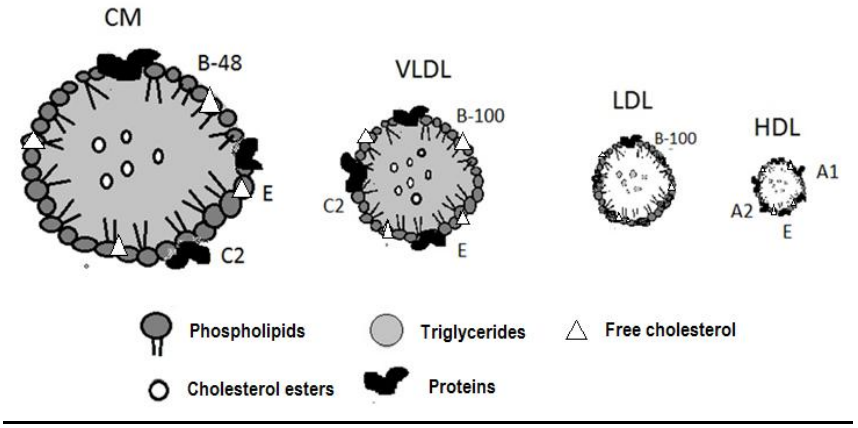

Figure 4. Lipoprotein structure. Lipoproteins contain a surface of phospholipid monolayer, cholesterol, and specific apolipoproteins (A,B,C,E). The core of CM and VLDL is TG rich, whereas LDL and HDL are rich in CE. CM and VLDL particle core contains approximately 70-90% TG and 3-10% CE (surface PL 5-10%). LDL particle core includes approximately 10% TG and 26% CE (surface PL 15%). HDL particle consists of 5% TG and 25% CE (surface PL 25%) (5).

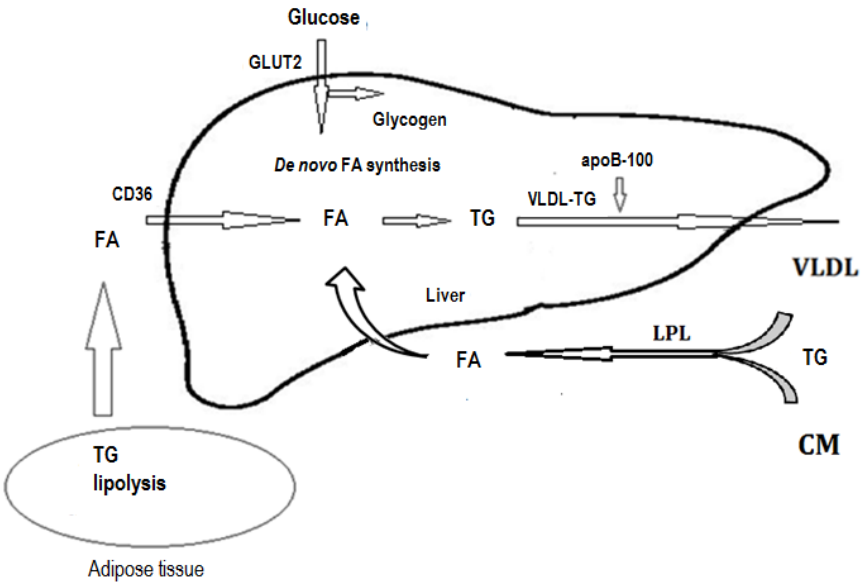

Figure 5. Hepatic VLDL synthesis. Liver synthesizes TG from fatty acids (FA) which are either imported or synthesized *de novo*. Fatty acids are stored as TG droplets and secreted into the bloodstream in VLDL lipoproteins.

2.1.4 Lipoprotein processing

CM and VLDL particles and their lipid content are processed in the bloodstream by multiple lipoprotein-modifying enzymes. TG in lipoproteins is hydrolysed by lipoprotein lipase (LPL), and the resulting fatty acids are taken up by tissues via fatty acid receptor CD36. Glycerol is taken up via aquaporin channels. During feeding triglycerides obtained from lipoproteins, and glucose, are primarily stored in WAT adipocytes (**Figure 6**) (6). After most of the TG is hydrolysed and internalized by tissues, remnant CM particles are taken up by the liver via LRP1 receptors. VLDL particles are transformed into cholesterol-rich LDL via intermediate-density lipoproteins (IDLs) by the function of hepatic lipase (HL). LDL particles enter cells via LDL receptors (LDLRs) (17-18), receptors whose abundance on the cell surface is regulated by the proprotein convertase subtilisin kexin (PCSK9) protease (54).

Besides VLDL, liver secretes nascent high density lipoprotein (HDL) particles, which harbor excess cholesterol and phospholipids from the surrounding tissues and from lipoproteins, to transport them back into the liver for degradation. Hepatic HDL uptake is mostly facilitated by scavenger receptor BI (**Figure 7**) (18). The HDL-mediated transfer of cholesterol into the liver is termed reverse cholesterol transport (RCT). HDL metabolism is influenced by lipoprotein-modifying enzymes CETP, PLTP, and LCAT. Cholesteryl ester transfer protein (CETP) facilitates the transport of cholesteryl esters and TG between HDL and TG-poor VLDL (17). The conversion of HDL particle into its mature form is supported by phospholipid transfer protein (PLTP) -mediated transfer of phospholipids to the HDL surface (17, 74) and by lecithin-cholesterol acyltransferase (LCAT), which converts free cholesterol from the cell surface into cholesteryl esters, which are then sequestered into the core of the HDL particle (**Figure 7**) (17).

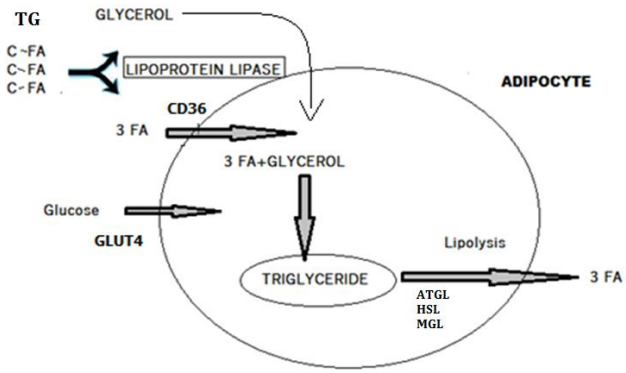

Figure 6. LPL function. Lipoprotein lipase breaks TG into glycerol and fatty acids, which are taken up by adipocytes via CD36 transporter and stored as TG during feeding. HSL-mediated lipolysis in the adipocytes results in the release of stored TG as fatty acids during fasting.

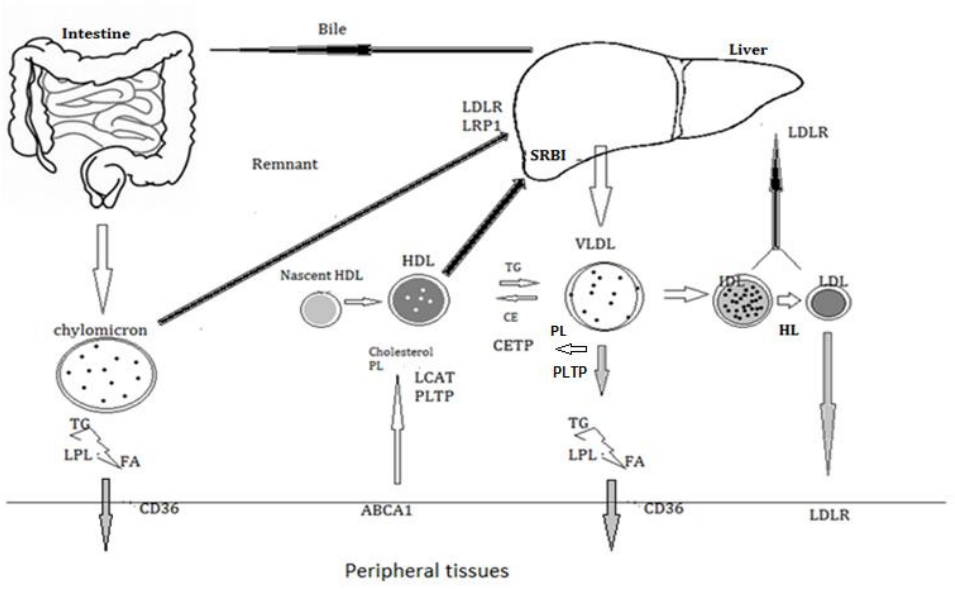

Figure 7. Lipoproteins in circulation. The small intestine and the liver secrete TG-rich CM and VLDL particles, which lose their TG core by LPL-mediated hydrolysis of TG into fatty acids. VLDL particles transform into cholesterol-rich LDLs, which are taken up by tissue via LDL receptors. Liver secretes HDL particles, which exchange CE and TG with VLDL particles via CETP. LCAT and PLTP facilitate the incorporation of PL and cholesterol components in the maturing HDL particle [figure modified from Lusis and Pajukanta (19)].

2.1.5 Insulin function

Insulin is the master regulator of blood glucose levels; its secretion from pancreatic beta cells is stimulated by high blood glucose during feeding. To maintain blood glucose at a stable level in the blood, insulin stimulates the uptake of glucose in the liver and muscle, and its metabolism to glycogen. Simultaneously, insulin decreases gluconeogenesis, the production of glucose from non-carbohydrate substrates. In the white adipose tissue, insulin influences the uptake of glucose and fatty acids and their conversion into triglycerides (20).

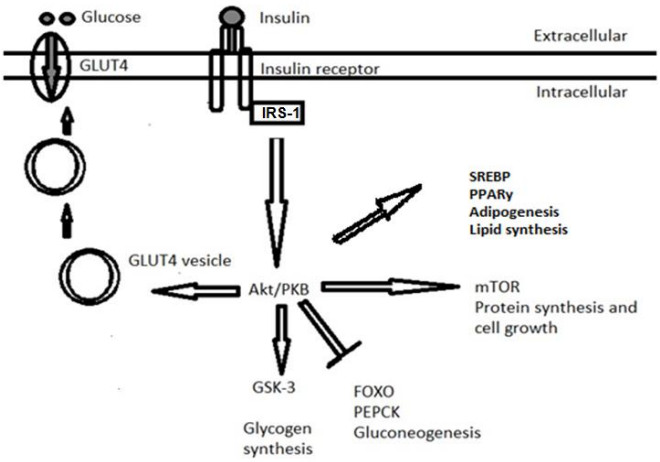

Figure 8. Insulin action. Insulin signal is mediated by AKT/PKB pathways that activate or suppress several metabolic processes within a cell, including increased glucose uptake via GLUT4.

When blood glucose level is low, pancreatic alpha cells secrete glucagon, which has the opposite effect of insulin; glycogen is converted back to glucose and excreted in blood, gluconeogenesis is increased, and TG lipolysis is activated in adipose tissue, resulting in a release of non-esterified fatty acids (NEFAs) into the bloodstream (**Figure 6**) (20). Lipolysis is facilitated by adipose TG lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL) (21).

The action of insulin is mediated by receptors – tyrosine kinases – on the surface of a cell. Once insulin binds to its receptor, activation of a signalling cascade ensues. Receptor binding activates an insulin receptor substrate 1 (IRS-1) and akt/protein kinase B (AKT/PKB), which ultimately induce a relocation of

glucose transporter type 4 (GLUT4) molecules into the cell membranes of adipose and muscle tissues, increasing the rate of glucose transport into these cells (20).

Insulin signalling also results in the activation of the enzyme glycogen synthase kinase 3 (GSK3), which causes liver and muscle cells to convert and store glucose into glycogen and inhibits the production and release of glucose by blocking gluconeogenesis and glycogenolysis. AKT/PKB also activates mammalian target of rapamycin (mTOR), which promotes protein synthesis (20). Insulin promotes the uptake of fatty acids and the synthesis of lipids in tissues via transcription factor steroid regulatory element-binding protein (SREBP), whilst inhibiting intracellular lipolysis (22). Reduced expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) during feeding decreases the transactivating potential of forkhead box protein O1 (FoxO1) and its target genes [such as phosphoenolpyruvate carboxykinase (PEPCK)] on the gluconeogenic pathways (23) (**Figure 8**).

2.1.6 Regulation of lipoprotein lipase

Lipoprotein lipase (LPL) shares structure and functions with two lipoprotein-modifying enzymes: hepatic lipase (HL) and endothelial lipase (EL). Liver-secreted HL has both phospholipase and TG lipase activities and plays an important role in HDL metabolism and in the conversion of VLDL to LDL **(Figure 7**). EL is expressed and secreted by endothelial cells and appears to be more specific in hydrolysing phospholipids and is more involved in HDL metabolism (24).

LPL is expressed in parenchymal cells and heart, muscle, and adipose tissue. LPL hydrolyses TG in lipoproteins into fatty acids, which are then rapidly taken up by tissues by fatty acid transporters (**Figures 6 and 7**). LPL is regulated by factors influencing the transport of the enzyme at its lipolytic site, where the enzyme interacts with glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1). LPL is also regulated by factors influencing its enzymatic activity at the site. Insulin activates LPL selectively in adipocytes, but not in the muscle, which ensures the guiding of fatty acids into the WAT during feeding. Muscle and myocardial LPL, in turn, is activated by glucagon and adrenaline (6).

ApoC-II located on the surface of CM and VLDL particles is needed for LPL activation, while apoC-III inhibits LPL activity (17).

ANGPTL proteins 3 and 4 regulate LPL activity during the fast-fed cycle. According to a model proposed by Zhang (25), feeding induces ANGPTL8, which through ANGPTL3 inhibits LPL in cardiac and skeletal muscle, thereby making circulating triglycerides available for uptake into the WAT. The reverse is true during fasting, when low insulin levels suppress ANGPTL8, but induce ANGPTL4 in the adipose tissue. This results in the inhibition of LPL in WAT and enhances uptake of TG-derived fatty acids in oxidative tissues such as skeletal muscle and heart (25).

2.2 ANGPTL3

2.2.1 Introduction to ANGPTL proteins

Angiopoietin-like proteins (ANGPTL) are a family of proteins with a structural similarity to vascular growth factors, angiopoietins. To date, eight ANGPTLs have been identified, all of which share similar protein structure: an amino-terminal coiled-coil domain (CCD), a linker region, and a carboxy-terminal fibrinogen-like domain (FLD). ANGPTL8, or lipasin, lacks the fibrinogen-like domain (**Figure 9**). Unlike angiopoietins, ANGPTLs do not bind TIE1 and 2 tyrosine kinase receptors, which regulate angiogenesis (26). ANGPTL3, -4, and -8 have been reported to have important roles in lipid metabolism.

ANGPTL3 is expressed primarily in the liver and to a lesser degree in the kidneys, muscle, intestine, and brain. ANGPTL4 is expressed in the same tissues as ANGPTL3, but unlike ANGPTL3, it is also expressed in the adipose tissue (27). ANGPTL8 expression is strongly induced by insulin and has the highest expression in the human liver, with much lower expression in white adipose tissue, brain, and lung (28).

ANGPTL3, -4, and -8 are proteolytically cleaved by proprotein convertases (**Figure 9**), which may have an important, yet not fully understood, role in the regulation of these proteins (27-28). ANGPTL3 enhances the inactivation of LPL by enhancing LPL cleaving facilitated by the endogenous furin and PACE4, which will dissociate LPL from the cell surface. The N-terminal part of ANGPTL3 is sufficient for LPL inactivation (29).

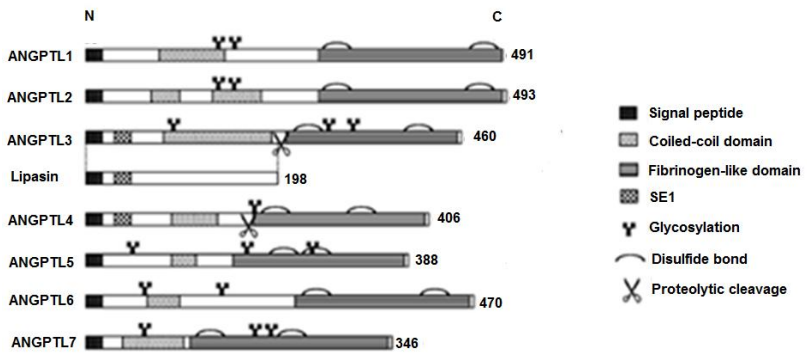

Figure 9. ANGPTL protein family. ANGPTL proteins share structural similarity, as they all possess a coiled-coil domain and fibrinogen-like domain, with the exception of lipasin (ANGPTL8), which lacks the fibrinogen-like domain. ANGPTL3 and ANGPTL4 are cleaved by proprotein convertases to separate the two domains, which may have important implications for their function in lipid metabolism (figure reprinted from Zhang et al. (30), with permission].

2.2.2 ANGPTL3 mouse models and genetic variants

The link between ANGPTL3 and lipid metabolism was first discovered in hypolipidemic mice that had spontaneously acquired a *de novo* mutation in *ANGPTL3*, resulting in an inactivation of the gene (31). The observed phenotype was followed by studies that demonstrated ANGPTL3 to have a functional role in binding and inactivating LPL (29,32).

Besides LPL, ANGPTL3 has been shown to affect EL activity, linking ANGPTL3 with HDL metabolism (33). No indications of ANGPTL3 with HL activity have been reported. Evidence of anti-atherogenic properties of ANGPTL3 deficiency emerged in apoE-knockout mice, which, due to high levels of TG and cholesterol, develop atherosclerotic lesions on a standard chow diet. When *ANGPTL3* LOF was introduced to APOE-KO mice, the amount of atherogenic lesions decreased (34). The mouse data were later supported by a genetic association analysis, which reported a decreased coronary heart disease (CHD) risk in subjects carrying LOF mutations in *ANGPTL3 (*35).

Homozygous carriers of *ANGPTL3* loss-of-function mutations, with undetectable levels of ANGPTL3 in plasma, develop familial combined hypolipidemia (FHBL2), a metabolic condition with very low plasma TG,

TC, LDL-C, and HDL-C levels (7, 8). Although rare, several FHBL2-causing genetic variants in *ANGPTL3* have been identified (**Table 1**), including *ANGPTL3* LOF s17*, which is common among the residents of the Italian village Campodimele (36). As demonstrated in Table 1, homozygote or compound heterozygote carriers of *ANGPTL3* LOFs manifest FHBL2, whereas lipid profiles of heterozygote carriers do not differ significantly from non-carriers.

Table 1. ANGPTL3 loss-of-function mutations and plasma lipid levels [adapted from Arca et al. (36)].

ANGPTL3 MUTATION	N	TC mmol/l	TG mmol/l	HDL-C mmol/l	LDL-C mmol/l	APOB (g/l)
HOMOZYGOTES						
S17X	**8**	2.4±0.3	0.4±0.1	0.8±0.2	1.3±0.4	0.5±0.1
E95del	**1**	1.4	0.3	0.3	1.0	0.3
N121LfsX2	**3**	1.7±0.5	0.4±0.1	0.3±0.1	1.2±0.3	0.4±0.1
N147X	**1**	1.4	0.8	0.4	0.6	NA
G400VfsX	**1**	1.8	0.4	0.6	1.2	0.5
COMPOUND HETEROZYGOTES						
S17x E129x	**4**	1.4±0.1	0.2±0.0	0.5±0.1	0.9±0.1	0.4±0.1
N147X F295L	**1**	1.5	0.3	0.5	0.9	NA
I19LFsX22 N147X	**3**	1.7±0.4	0.5±0.1	0.5±0.2	0.9±0.2	0.4±0.1
HETEROZYGOTES						
S17X	**72**	4.3±0.9	0.9±0.5	1.4±0.3	2.5±0.8	0.8±0.2
I19LFsX22	**3**	4.3±0.4	1.1±0.5	1.4±0.5	2.4±0.1	0.7±0.0
G56V	**1**	2.7	1.0	0.62	1.7	0.7
E96del	**2**	4.2±1.4	1.0±0.7	1.1±0.4	2.7±1.2	0.8±0.2
E119FsX8	**1**	2.1	0.4	0.6	1.3	0.3
N121FsX9	**1**	2.3	0.3	0.8	1.5	0.5
N121LFsX2	**4**	4.5±0.7	1.0±0.7	1.0±0.2	3.0±0.7	1.0±0.2
S122KFsX3	**1**	3.6	0.4	1.8	1.6	0.5
F129X	**6**	3.2±0.5	0.6±0.4	1.1±0.2	1.8±0.7	0.6±0.2
R332Q	**1**	1.6	0.8	0.6	0.5	NA
G400VFsX	**2**	4.7±0.0	0.8±0.2	1.5±0.6	2.7±0.7	0.8
Values expressed as mean±SD						

2.3 ATHEROSCLEROSIS AND DYSLIPIDEMIA

2.3.1 Atherosclerosis

Atherosclerosis is a condition identified by accumulation of cholesterol-enriched lesions (plaques) within the artery walls. Plaques may appear in any type of artery (heart, brain, pelvis, legs, arms, and kidneys) of the body and cause coronary heart disease (CHD), angina (chest pain), carotid artery disease (CAD), peripheral artery disease (PAD), or chronic kidney disease (CKD). A plaque may entirely block an artery and the blood flow into the heart or brain, resulting in a heart attack or stroke (37).

Atherosclerosis is the leading cause of cardiovascular disease and can be characterized as both a metabolic and inflammatory disease (38). The development of the condition may begin at an early age, remaining undetected due to the lack of symptoms until severe (37).

The development of a plaque is initiated by damage to the inner lining of the artery, called the endothelium. Because of the damage, triglyceride, cholesterol, platelets, cellular debris, and calcium accumulate over time in the artery wall. Monocytes, a type of white blood cells, recognize damaged endothelium and transform into macrophages, which, after engulfing oxidized or otherwise modified LDL particles, accumulate within the arterial intima as lipid-rich foam cells. Among foam cells, smooth muscle cells (SMCs) in the damaged area continue to divide and may form connective tissue. As a result, the arterial wall becomes markedly thickened by the accumulating cells and surrounding material (**Figure 10**). The arterial luminal space is then narrowed, reducing blood flow and oxygen supply (38).

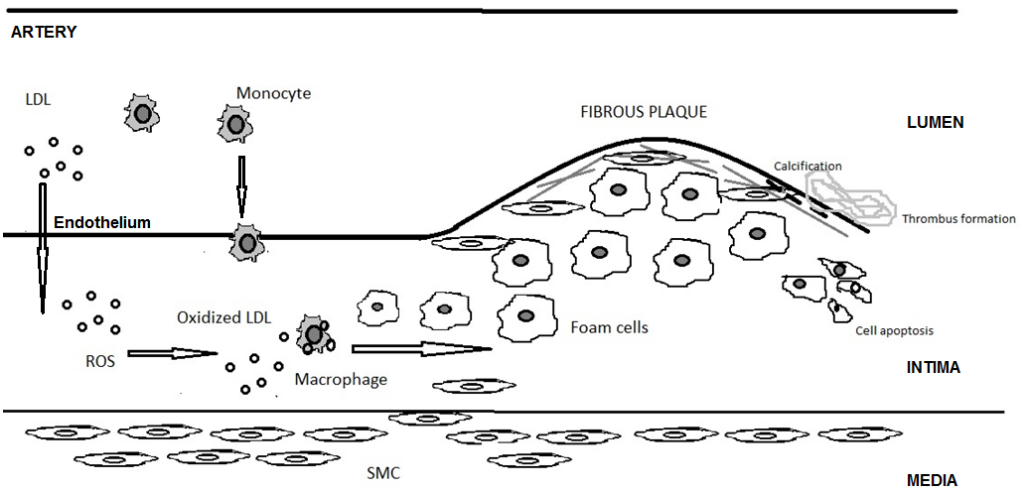

Figure 10. Mechanisms of atherosclerosis. LDL cholesterol is engulfed by macrophages which transform into lipid-rich foam cells in the arterial intima. The plaque formation is enhanced by smooth muscle cells (SMCs), which form connective tissue at the plaque site [figure adapted from Libby et al. (39)].

2.3.2 Dyslipidemia

Incidence rate

High cholesterol and triglyceride levels and low HDL in the blood are major risk factors for atherosclerotic cardiovascular disease (1). According to common knowledge, cardiovascular disease is rarely the result of a single monogenic cause and is likely a complex multifactorial disease, with a polygenic and environmental foundation (1). In Finland, one-third of the population has borderline high or high triglyceride and cholesterol values in the blood, which predicts high incidence rates for atherosclerotic cardiovascular disease (40).

In 1960, Finnish men had one of the highest death rates of coronary artery disease (CAD) in the world, a rate that has declined by 80% by 2000. Although the mortality rate of cardiovascular disease has declined, it remains the leading cause of death in the population (41, 42). In 2012, deaths due to heart failure and coronary artery events totaled 21 769 among all age groups (slightly over half of which were men) and 4052 among the working population (4/5 of which were men). On a population level, CAD is more common in eastern and northeastern Finland and among the lower socio-economic classes (43).

Common risk factors for atherosclerosis include age, male gender, obesity, diabetes, smoking, dyslipidemia, having close relatives with atherosclerosis at younger ages, and hypertension. Indicators of increased risk comprise high blood pressure (>140/90 mmHg), high BMI (>25), high glucose (>6 mmol/l), high TC (>5 mmol/l), high LDL-C (>3 mmol/l), and high TG (>2.26 mmol/l) (44).

Forms of dyslipidemia

Dyslipidemias rarely have a common genetic cause. The conditions are likely a combination of genetics (monogenic or polygenic) and lifestyle factors. Dyslipidemias due to a monogenic cause have been identified in genes such as (but not limited to) *LPL, APOE, APOC2, APOB, LDLR, PCSK9, ANGPTL3,* and *MTTP (45-48).*

Defects in LPL or apoC-II result in familial hyperchylomicronemia (FCS), which causes extremely high plasma TG levels due to impaired LPL function. Familial hypercholesterolemia (FH) and familial defective apoB-100 lead to high LDL-C due to defects in LDL receptor (LDLR), apoB and LDL-receptor adaptor protein 1 (LDLRAP1), or due to gain-of-function mutations in *PCSK9*. Familial dyslipoproteinemia caused by defects in apoE manifests as high TC and TG levels (45, 47).

No single monogenic factor has been identified to cause the two more common dyslipidemias (prevalence 1/50 to 1/100) familial hypertriglyceridemia and familial combined hyperlipidemia (FCH). In familial hypertriglyceridemia, TG and TC levels are high due to overproduction of VLDL. In FCH, TC, LDL, and/or TG are increased, while HDL is decreased. FCH affects 10-20% of the patients with premature CAD (**Table 2**) (45).

Hypolipidemias, such as hypobetalipoproteinemia (FHBL, low TC, and LDL-C), familial combined hypobetalipoproteinemia (FHBL2, low TC, TG, LDL-C, and HDL-C), and abetalipoproteinemia (no CM production), are caused by defects in apoB, ANGPTL3, or MTP (46).

Treatment of dyslipidemia

Hyperlipidemias are treated with drugs that target mainly TG, LDL-C, and HDL. The prevailing medications to correct dyslipidemias are statins in combination with niacin, fibrates, and fish oil (**Table 3**) (49).

Statins are the most commonly used therapeutic method to target LDL-C. Statins interfere with cholesterol synthesis, as the compound competitively inhibits the enzymatic activity of HMG reductase. HMG reductase reduces the rate by which the enzyme is able to produce mevalonate, an intermediate substrate within the cholesterol synthesis pathway. By blocking HMG reductase, liver can no longer produce cholesterol, leading to elevated LDL receptor synthesis, increased LDL uptake, and lower plasma TC (50).

Niacin's therapeutic effects are partly mediated through the activation of G protein-coupled receptors, including niacin receptor 1 (NIACR1) and niacin receptor 2 (NIACR2), which inhibit cyclic adenosine monophosphate (cAMP) production, and decrease the breakdown of TG in adipose tissue, and consequently, the release of free fatty acids in circulation. The reduced availability of fatty acids in the liver results in decreased production of TG and VLDL. Niacin treatment reduces TG levels by 30-50%, with less impact on LDL-C. The mechanism by which niacin increases HDL is unknown, but may be related to reduction in CETP mass and activity levels (51).

Fibrates reduce TG and LDL-C by stimulating cellular fatty acid uptake and their conversion into acyl-CoA derivatives, which are catabolised by the PPAR-mediated β-oxidation pathways. As a result, lack of available fatty acid substrates reduces hepatic TG and VLDL production. Increased HDL levels by fibrates may be caused by an induction of apoA-I and apoA-II expression (52).

Fish oil, rich in omega-3 fatty acids, reduces TG by 30-50%, with less impact on LDL-C or HDL. The mechanism by which fish oil reduces TG may be related to increased activity of LPL or decreased VLDL synthesis. Two mechanisms have been proposed by which VLDL synthesis is reduced: either omega-3 fatty acids may not be the preferred substrates for the enzyme diacylglycerol O-acyltransferase (DGAT) in TG synthesis, and/or omega 3 fatty acids may influence FA oxidation by interacting with nuclear transcription factors (53).

Table 2. Dyslipidemias.

Dyslipidemia	Defect	Genetic Cause
HYPERLIPIDEMIAS		
Familial hyperchylomicronemia (FCS)	Fasting triglycerides > 1000 mg/dl	Defect in LPL or apoC-II
Familial hypercholesterolemia	High LDL-C	Defect in LDL receptor, apoB, LDLRAP1, or PCSK9 gain of function (Prevalence 1:250-500)

Familial defective apo B 100	High LDL-C	Defective apoB (1:700)
Familial dyslipoproteinemia	Increased TC, VLDL, decreased HDL	Defect in apoE (1:5000)
Familial hypertriglyceridemia	Increased TC (due to VLDL) and TG, decreased LDL and HDL. Results from hepatic overproduction of VLDL.	Heterogeneous inheritance. Very sensitive to diet and EtOH. (Prevalence 1:50-1:100)
Familial combined hyperlipidemia	Increased TC, LDL, and/or triglycerides, decreased HDL.	Defects in HDL Metabolism. Heterogeneous inheritance. (Prevalence 1:50)
HYPOLIPIDEMIAS		
Hypobetalipoproteinemia	Homozygous patients have either shorter truncations, leading to lower TC and LDL-C, or absent apo B synthesis, leading to symptoms and signs of abetalipoproteinemia.	Defects in apoB and PCSK9. Autosomal dominant. (Prevalence 1:1000- 1:3000)
Familial combined hypobetalipoproteinemia	Low TG, LDL-C, HDL.	ANGPTL3 deficiency
Abetalipoproteinemia (Bassen-Kornzweig syndrome) chylomicron retention disease	Impaired CM production. ApoB deficiency Damage to central nervous system, poor growth	Defect in MTP

Table 3. Treatment for dyslipidemia [modified from Oh and Lanier (49)].

Substance	TG reduction %	LDL-C %	HDL (increase) %	Adverse health effects
Statin	20 to 40	18 to 55 reduction	5 to 15	Myopathy, rhabdomyolysis, elevated liver enzyme levels
Niacin	30 to 50	5 to 25 reduction	20 to 30	Flushing; worsening glycaemic control; elevated liver enzyme levels, especially with over the counter sustained-release niacin
Fibrates	40 to 60	5 to 30 increase	15 to 25	Rhabdomyolysis, especially with a gemfibrozil/statin combination
Fish oil	30 to 50	5 to 10 increase	5 to 10	Fishy after-taste, gastrointestinal upset

Novel treatments

Among the existing therapeutic approaches, alternative strategies to target plasma TG, TC, LDL-C, and HDL-C are being developed, including monoclonal antibody against LDL receptor inhibitor PCSK9 (54), monoclonal antibody, or antisense oligonuclotide technology (ASO) (55) -based inhibition against LPL inhibitors apoC-III (56) and ANGPTL3 (57) and Ezetimibe, which inhibits cholesterol absorption in the small intestine via inhibiting NPC1L1 function (58).

3 AIMS OF THE STUDIES

The three studies conducted approach ANGPTL3 function and related metabolism by utilizing different techniques in lipoprotein biochemistry, cell culture, and genetics.

In the first study, ANGPTL3 was silenced in lipoprotein-producing hepatocytes to determine how ANGPTL3 influences intracellular lipid metabolism and how expression and secretion of ANGPTL3 are regulated. The study was repeated in human enterocytes.

In the second study, the effects of a high-fat meal on lipoprotein metabolism in ANGPTL3 deficiency were investigated.

In the third study, genetic data on ANGPTL3 sequence variation was used to establish a link between ANGPTL3 variants and dyslipidemias in Finns. In another approach, a correlation analysis between ANGPTL3, ANGPTL4, or ANGPTL8 serum levels and lipid levels was conducted.

The studies focused on the following topics:

STUDY I: Molecular mechanisms of ANGPTL3 function: cell models

STUDY II: Clinical characterization of *ANGPTL3*-LOF carriers: postprandial lipoprotein response in humans

STUDY III: Finnish population study: *ANGPTL3* variants and ANGPTL serum levels

4 MATERIALS AND METHODS

4.1 Laboratory methods

Cell culture

Human liver hepatocytes immortalized by SV40 large T-Antigen (IHH, ATCC® PTA-5565™) and colorectal adenocarcinoma cells (Caco2, ATCC® HTB-37™) (**Figure 11**) were transduced with MISSION™ shRNA lentiviral vector particles (TRCN0000242782, Sigma Aldrich) targeting *ANGPTL3* (NM_014495.2) or with non-target shRNA (SHCOO2, Sigma Aldrich) (multiplicity of infection 1). Positive cells were selected with 5 µg/ml puromycin for 12 days. IHH cells were cultured in Williams' medium E (Gibco by Life Technologies, 22551-022) and Caco2 in DMEM (4.5 g/lglucose, BE12-614F, Lonza) with added 10% FBS and 0.2 mg/ml glutamine.

During experiments cells were cultured in 12-well plates in FBS-free media (5 mM glucose or 25 mM glucose and 0.33 mM oleic acid-BSA). Prior to experiments, Caco-2 cells were cultured on 12-transwell (pore size 3 µM) for 4 weeks to differentiate. Cells were washed with PBS (pH 7.4) and lysed in RIPA buffer. Protein concentration was measured with Bradford protein assay (Bio-Rad). The following compounds were used: Insulin (bovine, Sigma-Aldrich), Wortmannin (Sigma-Aldrich), Akt1/2 inhibitor

(Sigma-Aldrich), rosiglitazone (Cayman Chemical), and GW9662 (Sigma-Aldrich). Cell stocks were frozen in 10% DMSO-FBS at -70°C.

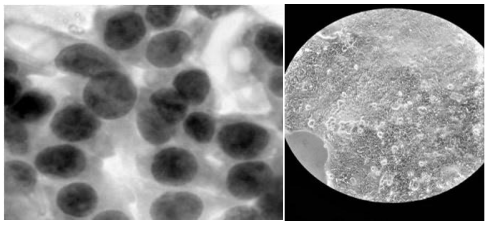

Figure 11. Human immortalized hepatocytes (A) and a monolayer of Caco2 enterocytes (B).

Protein measures

Commercial kits were used to measure plasma concentration of ANGPTL3 (dilution 1/500), ANGPTL4 (dilution 1/5) (Human Angiopoietin-like 3 DuoSet ELISA DY3829 and Human Angiopoietin-like 4 DuoSet ELISA DY3485; R&D Systems, MN, USA), adiponectin (Human Adiponectin Platinum ELISA, Affymetrix, eBioscience, San Diego, USA), leptin (Laboratorni Medicina a.s, Brno, Czech Republic), fatty acid binding protein 4 (FABP4) (Laboratorni Medicina a.s, Brno, Czech Republic), apoB-100 (Mabtech), apoB-48 (Shibayagi), and apoC-III (Assaypro LLC, Missouri, USA). PLTP and CETP activity measurements and ANGTL8 ELISA are described in Study III.

BSA-oleic acid complex

BSA bound oleic acid (0.5%, 1 mM OA) solution was prepared as follows: BSA (0.125, defatted, Sigma-Aldrich) was diluted in 25 ml of DMEM. NaOH was used to raise the pH of the solution to 10. Oleic acid (30 mg, sodium oleate, Sigma-Aldrich) was diluted in 1 ml of sterile H_20 at 37°C. Albumin solution was enriched with 250 µl of oleic acid solution to form BSA-oleic acid complexes. Solution pH was decreased to 7.7 with HCl. During experiments the oleic-acid-BSA solution was used in 1/3 dilution (0.33 mM).

Thin-layer chromatography

Cells were incubated in the presence of 5.5 mM glucose and 0.5% BSA-complexed with 0.373 mM oleic acid and 0.1 uCi [14C]-oleic acid (Perkin Elmer) for 24 h. Cells were collected in 1 ml of 2% NaCl-PBS buffer. Lipids were extracted by adding 2 ml of methanol and 1 ml of chloroform and centrifuged (2500 rpm 10 min RT). H_2O (1 ml) and chloroform (1 ml) were added and centrifuged (2500 rpm, 10 min at room temperature). The upper phase was removed and the lower phase dried under nitrogen and solubilized in 100 µl of chloroform and applied on a TLC-plate. TLC-plates were transferred to a chamber including N-hexane, diethylether, acetic acid, and H20 (65/15/1/0.25, respectively v/v). Iodine vapour-stained areas containing triglycerides were scraped out from the TLC-plate, and radioactivity was measured by liquid scintillation counting (Wallac LS-Beta-Counter, Turku, Finland).

FPLC fractionation

Aliquots (500 µl) of pooled plasma obtained from FHBL2 and control subjects were applied on a Superose 6HR size-exclusion chromatography column (Pharmacia Biotech) at a flow rate of 0.5 ml/min in PBS (pH 7.4) containing 1 mM EDTA. Fractions (0.5 ml) were collected and analysed for TG, TC, and PL concentration by enzymatic methods described in the lipid measures section below. TG-rich lipoprotein (TRL) particles (chylomicrons, VLDL and VLDL remnants) were eluted together in the void volume of the column.

Lipid measures

Triglycerides from media and cell lysates were measured with an enzymatic method (GPO-PAP 1488872 kit; Roche Diagnostics Gmbh). Phospholipids were measured from the cell culture media with an enzymatic method (FS 60080970; Diasys Diagnostics). Plasma FFAs were measured by an enzymatic colorimetric assay (Wako Diagnostics).

Measurement of glucose uptake

Cells were incubated in the presence of 5.5 or 20 mM glucose and 1 µCi of deoxy-D-glucose, 2-[1,2-3H (N)] (1 mCi/ml, Perkin Elmer). Radioactivity from cell lysates and media was measured by liquid scintillation counting (Wallac LS-Beta-Counter, Turku Finland).

Q-PCR analysis

RNA was extracted with a Qiagen RNA purification kit and synthesized into cDNA using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies). Each qPCR well contained primers at 300 nM concentration and 10 ng of cDNA. The total volume of each reaction was 10 µl containing SYBR® Green PCR Master Mix (Life Technologies). The qPCR program was as follows: 95°C for 10 min and 45 cycles of 95°C for 15 sec and 60°C for 1 min.

Western blot

Cells from 6-well plates were scraped in 500 ul of PBS and centrifuged 2000 rpm for 5 min. After removing the supernatant, the cells were lysed in 30 ul of ripa buffer with protease inhibitors (Sigma-Aldrich). Gradient SDS-PAGE gels (Trupage, Sigma-Aldrich, 4-12%, 12-wells) were loaded with 50-100 µg of protein. Gels were blotted for 1.5 h at 400 mA. Membranes were blocked in 5% milk-TBST for 1 h. Antibodies were diluted in 0.5% milk-TBST (antibody dilutions: GLUT2 1/500, CD36 1/100, ACTIN 1/1000, Abcam) and incubated overnight. Secondary antibodies were added for 1-2 h (goat antimouse and goat antirabbit, 1/1000 dilution). Between antibody treatments, membranes were washed in TBST for 1 h.

Statistics

Cell experiments were conducted in triplicate (N=3) and performed with 2-3 separately transduced cell lines. Results are expressed as mean +/- SD. The difference between groups was considered significant when $P<0.05$ using Student's T test (*<0.05, **<0.01, ***<0.001).

Method summary

Studies:

I. *ANGPTL3*-silenced hepatocytes and enterocytes

II. Postprandial lipid response in ANGPTL3-deficiency

III. Finnish ANGPTL3 variants and serum ANGPTL levels

LABORATORY METHODS	STUDY (I-III)
Cell culture:	
Lentiviral silencing with shRNA	I
Light microscopy	I
Labelled substrates and scintillation counting	I
Protein measures and enzyme activities:	
ELISA assays for quantitative protein analysis	I-III
Western blot	I
RNA and DNA:	
Quantitative PCR	I
Gene sequencing	III
Whole-exome sequencing data	III
Lipid methods:	
Lipid extraction and thin-layer chromatography	I
Enzymatic lipid assays to measure tg, cholesterol, PL, NEFA	I-III
FPLC: lipoprotein fractionation	II
Statistical analysis, software	
EXCEL	I-III
SPSS	II,III
Ensembl VEP	III
NCBI snp	III
Panther Pathway	III

4.2 Study populations

Population in Study II: ANGPTL3-deficient subjects derived from the village of Campodimele, Italy. The population comprised carriers of p.S17* LOF mutation in the ANGPTL3 (5 homozygotes, 29 heterozygotes)

and 35 control subjects who did not carry any ANGPTL3 variants. Two compound heterozygous and 2 heterozygous subjects carrying the inactivating ANGPTL3 p.E94* and p.N121KFs*3 mutations were additionally recruited from a family manifesting the FHBL2 phenotype. None of the subjects consumed special diets or took vitamins, antioxidants, or medications affecting lipid metabolism. All participants were invited in the morning after overnight fast to receive clinical evaluation, blood drawings, body composition and indirect calorimetry measurements, and oral fat tolerance test (OFTT).

Populations in Study III: Subjects who carried ANGPTL3 SNP variants were selected among the FINRISK study population, with available whole-exome sequence data produced by the Finnish Institute for Molecular Medicine (FIMM). The FINRISK study data were collected in several districts from 25- to 74-year-old Finns (N=38,000) during 1992-2012 by the National Institute for Health and Welfare. Whole-exome sequencing data were available for 6,069 FINRISK subjects (59-61).

Subjects for the ANGPTL3 serum level study were selected from the Health 2000 survey. Subjects whose plasma TG and cholesterol were at the lower or higher end were chosen for the analysis (30 hypolipidemic and 34 hyperlipidemic subjects). The Health 2000 health examination survey was a large epidemiological health survey performed in Finland from fall 2000 to spring 2001 and included 6,771 participants representing the Finnish population aged ≥30 years. The sample was drawn from 80 health service districts throughout Finland using a two-stage cluster sampling (62).

5 RESULTS:

5.1 STUDY I: ANGPTL3-silenced hepatocytes

Intracellular function of ANGPTL3 was investigated in lipoprotein-producing human immortalized hepatocytes (IHH) by silencing *ANGPTL3* expression with Lenti-shRNA. *ANGPTL3* expression and secretion were decreased dose-dependently by rosiglitazone and insulin in the non-silenced cells (Study I, Figure 1 C, 3 B).

Insulin stimulation reduced the secretion of TG and PL in silenced cells near to the level of TG secreted by the control cells, with a smaller decrease in secreted apoB-100 levels. Therefore, the TG/apoB-100 ratio was more dramatically reduced in *ANGPTL3*-silenced cells (**Figure 12 A**). These findings suggest that the silencing of *ANGPTL3* results in a more pronounced shift from the secretion of large TG-enriched VLDL1-type particles to small lipid-poor VLDL2-type particles during insulin stimulation (feeding), which may also reflect improved insulin sensitivity. When substrate uptake was investigated with labeled substrates, *ANGPTL3*-silenced cells showed a 45% increase in glucose uptake in the absence of insulin (**Figure 12 B**). No changes in fatty acid uptake were observed (Study I, Figure 7 B).

Gene expression analysis did not show any significant alterations in gene expression levels of *ANGPTL8*, *ANGPTL4* , *IRS-2*, *GLUT2* , *APOAI, APOB, FAS, DGAT1, LXR, SREBP1C, PTEN, PPARG (PPAR-y), IL6, HNF4*α, and *PI3K* (regulatory subunit p85) (Study I, Figure 8). Expression of peroxisome proliferator-activated receptor γ co-activator 1-α (*PGC1α*) and its downstream targets phosphoenolpyruvate carboxy-kinase (*PEPCK*) and tribbles homologue 3 (*TRB-3)* were significantly down-regulated in *ANGPTL3*-silenced

cells, suggesting that a down-regulation of gluconeogenic genes was linked to the increased glucose uptake observed (Study I, Figure 8).

As expected, fatty acid (oleic acid) treatment increased the expression of *CD36* fatty acid transporter in control cells, but significantly less in the *ANGPTL3*-silenced cells. *PLTP* expression was lower in *ANGPTL3*-silenced cells and was significant in glucose-treated cells (**Figure 13 A, B**). In a Western blot analysis, the protein level of CD36 was reduced in the silenced cell line, whereas the protein level of GLUT2 was increased (**Figure 13 C**).

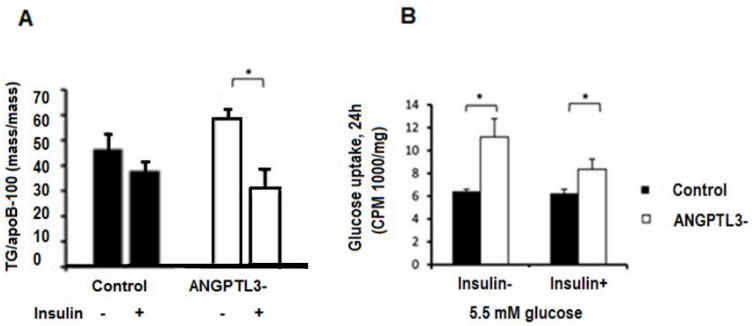

Figure 12. TG/ apoB-100 ratio and glucose uptake in *ANGPTL3* silenced vs. control cells. TG/apoB-100 ratio was more dramatically reduced in *ANGPTL3*-silenced cells by insulin [47%, from 60 to 32 (mass/mass)] than in control cells [16%, from 45 to 38 (mass/mass), **12 A**]. ANGPTL3-silenced cells showed a 45% increase in glucose uptake (measured by a non-hydrolysable radiotracer, [^{3}H]-labelled deoxy-D-glucose) in the absence of insulin (**1 B**), p *<0.05, **<0.01, ***<0.001. [Figures reprinted from Tikka et al. (Study I) with permission].

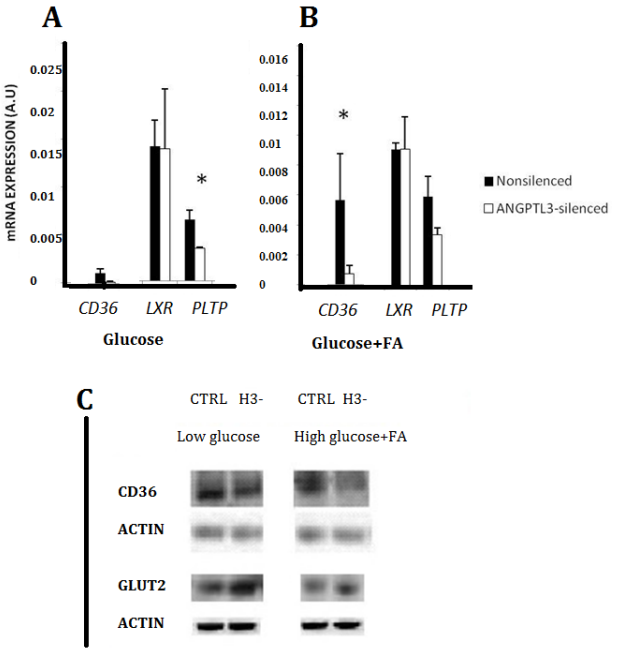

Figure 13. Gene expression and Western blot analysis of *ANGPTL3* silenced vs. control cells

FA-treatment (0.33 µM oleic acid) increases the expression of *CD36* fatty acid transporter in hepatocytes, but significantly less in *ANGPTL3*-silenced cells than in control cells. *PLTP* expression is significantly lower in *ANGPTL3*-silenced cells, whereas *LXRα* expression remains unchanged (A-B). Western blot analysis shows higher protein mass of CD36, but lower GLUT2 mass in non-silenced (CTRL) cells than in *ANGPTL3*-silenced cells (H3-) during both low glucose and high-glucose+FA treatment (C), p *<0.05, **<0.01, ***<0.001 (unpublished data).

Gene expression data suggest that the silencing of *ANGPTL3* leads to a shift in substrate utilization between fatty acids and glucose, in favour of glucose. Reduced *PLTP* expression hypothetically will manifest as decreased PLTP protein mass, which may have implications in HDL metabolism.

5.2 ANGPTL3-silenced enterocytes (unpublished data)

Intracellular function of ANGPTL3 was studied in lipoprotein-producing human Caco2 enterocytes by silencing *ANGPTL3* with Lenti-shRNA. ANGPTL3 secretion was nearly non-existent in *ANGPTL3*-silenced cells. Rosiglitazone decreased ANGPTL3 secretion by 75%, while LXR agonist T090 increased ANGPTL3 secretion by 65% and LXR antagonist GSK or insulin had no impact on ANGPTL3 secretion (**Figure 14**). The mRNA expression pattern of *ANGPTL3*-silenced cells displayed downregulation of *CD36,* which was significant in FA (oleic acid) -treated cells (**Figure 15**). Despite downregulation of fatty acid transporter *CD36* expression, no differences in oleic acid uptake were observed in *ANGPTL3*-silenced cells compared with non-silenced cells. Silencing did not affect apoB-48 or TG secretion.

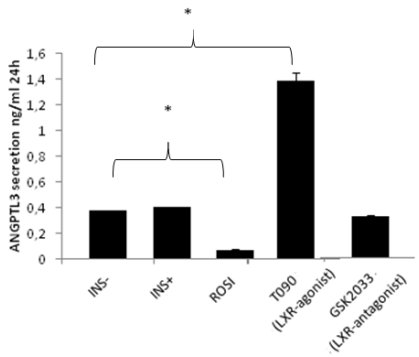

Figure 14. ANGPTL3 secretion in Caco2 enterocytes. ANGPTL3 secretion is significantly reduced by rosiglitazone treatment (50 µm) and significantly increased by T090 treatment (LXR agonist, 10 µm). Treatment with GSK2033 (10 µM) or insulin (100 nM) had no impact on ANGPTL3 secretion compared with non-treated cells, p *<0.05, **<0.01, ***<0.001 (unpublished data).

Based on the data, we concluded that expression of *ANGPTL3* is regulated by LXR and PPARγ agonists in hepatocytes and enterocytes and that silencing of *ANGPTL3* in Caco2 enterocytes caused a similar downregulation of *CD36* transporter as seen in silenced hepatocytes, although, as in hepatocytes, no differences in fatty acid (oleic acid) uptake were observed. No significant alterations were detected in secretion of TG or apoB-48 (data not shown), indicating that the silencing does not affect CM secretion in these cells.

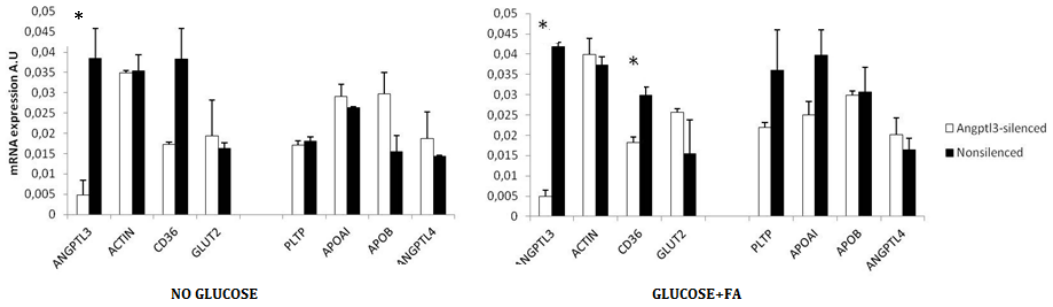

Figure 15. Expression pattern of *ANGPTL3*-silenced enterocytes and control cells. *CD36* expression is significantly decreased in glucose+ FA (0.33 µm oleic acid) treated enterocytes, p*<0.05, **<0.01, ***<0.001. (unpublished data).

5.3 STUDY II: Postprandial response in ANGPTL3-deficient subjects

Subjects who were either homozygotes (S17*, N=5) or compound heterozygotes (E94* and p.N121KFs*, N=2) for ANGPTL3 LOF or heterozygote carriers of ANGPTL3 LOF (S17*, N=29) were compared with a control population (with no inactivating mutations in *ANGPTL3*, N=35) in a study where subjects were given a high-fat meal followed by measurements of plasma lipid and lipoprotein levels and other relevant metabolic biomarkers.

5.3.1 ANGPTL3, NEFA, and glucose levels

Homozygote carriers for ANGPTL3 LOFs have no circulating ANGPTL3 and heterozygotes have approximately 51% less circulating ANGPTL3 than control subjects. In heterozygous and control subjects, ANGPTL3 plasma levels show a significant decline (24% and 23%, respectively, at 6 h) during the postprandial timeline of 6 h relative to fasting values (**Figure 16 A**).

Plasma non-esterified fatty acid levels (NEFAs) were 36% lower in ANGPTL3-deficient subjects during fasting and after 2 h in a postprandial sample than in heterozygotes and control subjects. NEFA levels showed a 45% increase from baseline in control subjects, a 33% increase in heterozygotes, and a 98% increase in homozygotes at 6 h (**Figure 16 B**). Both plasma glucose and insulin levels increased in all groups, reaching peak values at 2 h and returning to basal levels at 6 h.

β-hydroxybutyrate (β-HBA), a “ketone body” produced in the liver by fatty acid oxidation, was measured in the plasma of subjects to determine whether lower NEFA yielded lower β-HBA levels in homozygotes. Fasting levels of β-HBA did not differ between subjects. β-HBA peaked at 6 h with a percentage increase significantly lower in homozygotes (0.033 ± 0.063 mmol/L and +36%, respectively) than in heterozygotes (0.046 ± 0.085 mmol/L and +49%, respectively) and controls (0.062 ± 0.076 mmol/L and +68%, respectively, p<0.05).

5.3.2 Postprandial lipid biomarkers

In total, homozygotes had 70% less TG in circulation than control subjects during the postprandial phase. Total TG values in heterozygotes did not significantly differ from control subjects. TG values peaked at 4 h, and the increase in TG compared with baseline values was 73% in control subjects and 25% in homozygotes. In heterozygotes, TG values peaked at 2 h, with a 75% increase relative to baseline values (**Figure 17 A**).

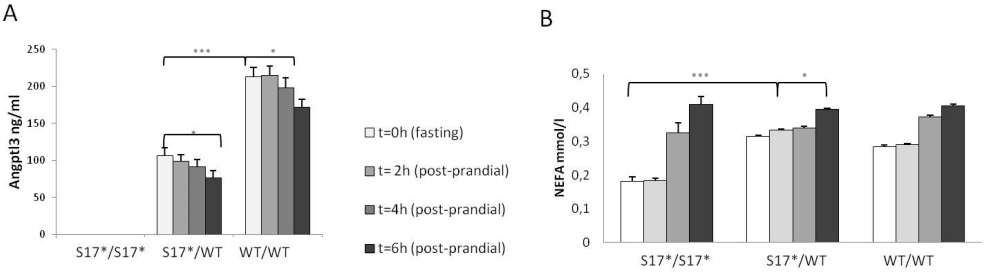

Figure 16. ANGPTL3 levels and NEFA levels. Homozygote carriers of ANGPTL3 LOFs have no circulating ANGPTL3. Heterozygote LOF carriers and control subjects show a steady decline in ANGPTL3 plasma levels during the postprandial period **(A**). Plasma NEFA levels are significantly lower in homozygotes at fasting and at 2 h (**B**), p *<0.05, **<0.01, ***<0.001 (modified from Study II, Figure 3A, 4A).

In total, homozygotes had 78% less apoB-48 in circulation than control subjects. Heterozygote subjects showed intermediate values, with 35% less apoB-48 than control subjects. Much like TG, plasma apoB-48 levels peaked at 4 h, with a 126% increase in control subjects and an 87% increase in homozygous subjects relative to baseline values. In heterozygotes, apoB-48 peaked at 2 h, with a 66% increase from the baseline value (**Figure 17 B**).

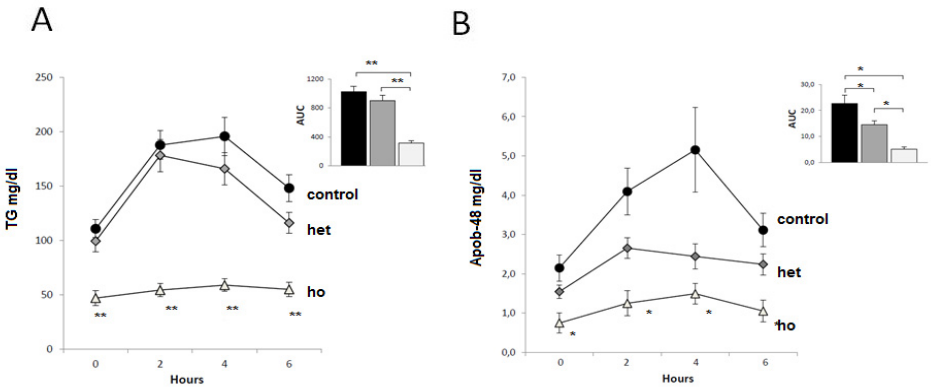

Figure 17. TG and apoB-48 levels at baseline and at 2, 4, and 6 hours after a high-fat meal. Homozygote ANGPTL3 LOF carriers (ho) demonstrate a 70% reduction in total TG and a 78% reduction in apoB-48 (AUC, area under the curve) compared with control subjects (control). Heterozygote (he) LOF carrier values for total apoB-48 are intermediate, p*<0.05, **<0.01, ***<0.001. [Figure reprinted from Minicocci et al. (Study II) with permission].

Baseline TC values were 59% lower and apoB-100 values 42% lower in homozygotes than in control subjects and were not changed in the postprandial samples. The data indicate that the clearance of CM particles is very rapid in homozygotes relative to control subjects, with a 70-78% decrease in total circulating TG and apoB-48. Heterozygotes show an intermediate values in apoB-48 and a less pronounced change in total TG.

5.3.3 Other metabolic biomarkers (unpublished data)

Adiponectin, FABP4, or leptin did not show any significant difference between study groups. Adiponectin levels declined significantly in postprandial samples after 2 h and remained low at 6 h (**Figure 18 A**), whereas FABP4 levels declined significantly after 4 h and recovered to the baseline level at 6 h (**Figure 18 B**). Leptin levels did not show significant fluctuations from baseline up to 6 h (**Figure 18 C**). Plasma leptin levels correlated with gender, being significantly higher in females (**Figure 18 D**). PLTP activity showed a trend towards lower values in s17* homozygotes (**Figure 18 E**).

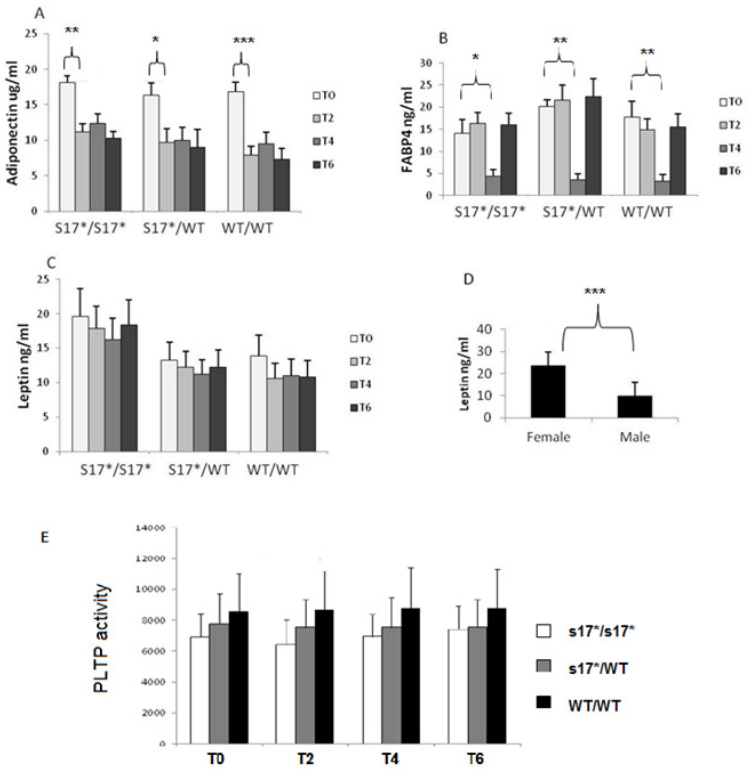

Figure 18. Other metabolic markers in postprandial samples. Fasting values and values at 2, 4, and 6 hours (T0-T6) of adiponectin, FABP4, and leptin (**A-C**). Leptin correlates with gender, with higher values in females (**D**). PLTP activity (nmol/ml/h) shows a trend towards lower values in s17* homozygotes in each time point (**E**). WT= wild-type/ control subjects, p*<0.05, **<0.01, ***<0.001 (A-D unpublished data, E modified from Study II, Supplement 3).

5.4 STUDY III: Finnish *ANGPTL3* variants and ANGPTL3 serum levels

To compare the correlation between ANGPTL3 serum levels and lipid and lipoprotein levels, and other biomarkers, ANGPTL3, -4, and -8 and apoC-III were measured in serum samples of hypolipidemic and hyperlipidemic subjects. Serum ANGPTL3, - 4, or -8 levels did not correlate with any of the lipid or lipoprotein attributes tested (**Table 4**). ANGPTL3 correlated positively with age and PLTP activity. Also ANGPTL3 and ANGPTL8 positively correlated with CETP activity (Study III, Table 1).

Table 4. Lipid, apoC-III, and ANGPTL- protein concentrations in hypolipidemic and hyperlipidemic groups.

Subjects	TG	TC	LDL-C	HDL-C	APOC-III	ANGPTL3	ANGPTL4	ANGPTL8*
HYPOLIPIDEMIC (N=30)	0.55±0.072	3.77±0.50	1.86±0.43	1.55±0.28	151± 50	201.57±70.52	362.16±213.3	1.89±2.53
HYPERLIPIDEMIC (N=34)	3.87± 0.80	7.00±0.84	4.15±0.88	1.08±0.24	325± 56	222.40±110.7	300.12±185.6	2.12±4.62
***P*-value**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.375	0.191	0.196

*Mean ±SD. Lipids are expressed as mmol/l, apoC-III as (µg/ml), ANGPTL3 and 4 as (ng/ml), and ANGPTL8 as (µg/ml).

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In the genetic study, ANGPTL3 variants identified among the 6,069 whole-exome sequenced FINRISK subjects were matched with their lipid profiles. ANGPTL3 exome sequence was relatively conserved among the population, showing little variance. Among the 8 *ANGPTL3* variants identified, one subject with the variant rs199772471 and 4 subjects with the variant rs12563308 had abnormally high TC and LDL-C (**Table 5**). Rs12563308 is a synonymous variant and was therefore not considered harmful in SIFT or polyphen-2 predictions, based on which we concluded that the phenotype may be caused by another genetic factor, one which is possibly shared by these 4 subjects.

Table 5. *ANGPTL3* variants with abnormal TC and LDL-C values.

Rs-number, Ref, Alt, Alt Fq, N*	AGE	BMI	APOAI	APOB	HDL-C	TC	LDL-C	TG**
rs12563308, T, C, 0.00034, 4	54.75±17	28.80± 4.0	1.80± 0.3	1.41± 0.3	1.92± 0.5	**7.99± 1.3**	**5.08± 1.3**	1.17±0.4
rs199772471,G,A, 0.00005, 1	46	22.23	1.67	1.51	1.48	**7.93**	**5.22**	2.04
Control group	45.99± 12	26.7± 4.8	1.63± 0.3	1.01± 0.3	1.41± 0.4	5.55± 1.1	3.46± 0.9	1.52± 1.1

*Ref: reference allele, Alt: alternative allele, Alt Fq: frequency of alt allele, N= number of heterozygous carriers

**Mean ±SD. Lipids are expressed as mmol/l, apoA-I and apo-B as mg/ml

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When whole-exome sequence data from the 4 variant carriers (and from the carrier of rs199772471) were analyzed in more detail, no obvious monogenic cause (such as LOFs in LDLR) was found. In the second

phase the following criteria was used to screen for variants in the whole exome sequencing data: the variant is rare (frequency less than 0.1), is in a gene involved in cholesterol metabolic pathway, and is considered harmful (according to SIFT&polyphen-2 predictions). As a result 4 candidate genes were identified, and homozygous carriers of these variants were matched with their lipid profiles (**Table 6**).

As demonstrated in **Table 6,** one variant in *STARD3* (rs11556624) was associated with abnormally high TC and LDL-C in both (N=2) homozygous carriers (no data on heterozygotes).

Table 6. Lipid profiles of homozygote carriers of potentially harmful and rare variants in the genes involved in cholesterol metabolism.

GENE (VARIANT)	N (1/1)	TG*	LDL-C	HDL-C	TC*
ALOX12 (rs147158964)	3	2.19±1.39	4.19±1,22	1.66±0.3	7.11±1.19
SLC2A8 (rs34064803)	24	1.37±0.79	3.72±0.76	1.47±0.3	5.93±0.87
PAQR6 (rs7513351)	41	1.51±1.18	3.44±0.93	1.46±0.38	5.63±0.95
STARD3 (rs11556624)	2	1.82±0.37	**6.38±1.08**	1.27±0.07	**8.73±1.2**
REFERENCE VALUE	300	1.52±1.08	3.46± 0.92	1.41± 0.38	5.55± 1.05

*SD±mean. The unit is mmol/l.

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6 DISCUSSION

VLDL and CM secretion

The postprandial response to a high-fat diet in ANGPTL3-deficient subjects indicates that the clearance of TG from the circulation is extremely efficient compared with control subjects, who show a typical response to a high-fat meal with increased TG and apoB-48 values. Based on the data, we suggest that the low TG and apoB-48 values in ANGPTL3 deficiency are linked to increased activity of LPL and a faster clearance rate of lipoprotein remnants from the circulation. This assumption is based on the fact that FHBL2 subjects do not show signs of steatorrhoea or have lower BMI or fat mass, signs which may indicate impaired CM production. Also, as demonstrated in the cell culture experiments, silencing of *ANGPTL3* did not decrease the secretion of apoB-48 or TG in *ANGPTL3*-silenced enterocytes. A reduction in total cholesterol and apoB-100 levels in FHBL2 subjects may be due to liver-specific downregulation of VLDL lipidation and secretion (8, 63), which may be coupled with faster clearance of VLDL/IDL remnants. In animal experiments, mice treated with ANGPTL3-inactivating antibody showed decreased TC levels even when LDL-receptor and LRP1 were blocked, indicating that the low cholesterol levels in ANGPTL3-deficiency are not a result of increased LDL receptor activity (63), but rather reduced lipidation and secretion of VLDL.

In the liver, LXRs are shown to activate triglyceride synthesis directly via SREBP1c, which in mice leads to increased VLDL secretion and hypertriglyceridemia (64). As shown in cell culture experiments, LXR agonist T0901317 is a strong inducer of ANGPTL3 expression, indicating that ANGPTL3 is a downstream target of LXRα in both hepatocytes and enterocytes. Therefore, increased VLDL secretion and hypertriglyceridemia

are linked to LXR induction, which in turn increases ANGPTL3 expression and secretion. On the contrary, low LXRα levels and its downstream target *ANGPTL3* could be linked to hypolipidemic states in lipoprotein-producing cells (65).

Substrate utilization

As reported in the postprandial study, ANGPTL3-deficient subjects show lower NEFA levels in plasma during fasting. Low fasting NEFA levels in ANGPTL3 deficiency may be a manifestation of blunted activation of lipolysis in the adipose tissue, which is supported by studies showing that ANGPTL3 induces lipolysis in WAT (66). On the basis of these observations, we suggest that the lack of fatty acid substrates from the adipose tissue directed into the liver may cause decreased synthesis and secretion of VLDL and contribute to hypolipidemia in ANGPTL3 deficiency. In the cell culture experiments, silencing of *ANGPTL3* caused a stable reduction in fatty acid transporter CD36 and increased glucose uptake; alterations that may reflect depletion in the supply of FA into the liver. In addition, ANGPTL3-deficient subjects showed lower concentrations of β-HBA towards the end of the postprandial period, which is an indication of reduced fatty acid oxidation in the liver (Study II).

Insulin sensitivity

An accumulating amount of data indicates that insulin down-regulates the expression and secretion of ANGPTL3 in a dose-dependent manner (67), which was also observed in cell culture experiments and in the postprandial study with ANGPTL3-deficient subjects (Studies I and II). PPARγ is a downstream target of insulin, and has been shown to be activated during adipogenesis (68). Rosiglitazone, a PPARγ agonist, has previously been used as a drug against insulin resistance since it is able to activate some of the insulin-induced responses in the adipose tissue and liver (68). As shown in the cell culture experiments (Study I), rosiglitazone decreased the expression and secretion of ANGPTL3 in a dose-dependent manner, an observation that links low ANGPTL3 levels to activation of insulin-induced pathways. ANGPTL3-deficient subjects may have enhanced insulin sensitivity, as suggested by their HOMA-IR index, and decreased prevalence of type 2 diabetes mellitus (69-70). In line with these observations, increased ANGPTL3 serum levels have been reported in diabetic patients (71-72). In cell culture experiments (Study I), *ANGPTL3*-silenced hepatocytes displayed more reactivity towards insulin treatment than the control cells, as the insulin treatment resulted in a decreased lipidation of apoB-100-containing particles in the silenced cells.

ANGPTL3 serum levels and activity

As shown in study III, ANGPTL3 plasma levels did not correlate with plasma lipids or lipoprotein levels in hypolipidemic and hyperlipidemic subjects, a result also apparent in a larger population sample (73). A 50% reduction in circulating ANGPTL3, in *ANGPTL3* LOF S17* heterozygotes (Study II), does not have a dramatic impact on the lipid profiles of these subjects compared to control subjects. Therefore, it seems that ANGPTL3 is not only regulated by its concentration (and secretion rate), but also by its activity state in circulation. The active state may be related to the cleaving of ANGPTL3, as the N-terminal half of the protein is sufficient to inhibit LPL. ANGPTL8 is proposed to be involved in the cleaving process (25); however, recent reports indicate that ANGPTL3 can inhibit LPL independent of ANGPTL8 (74).

HDL and lipoprotein-modifying enzymes

Even though ANGPTL3 plasma levels did not correlate with plasma lipids or lipoprotein levels in hypolipidemic or hyperlipidemic subjects, positive correlations with lipoprotein-modifying enzymes PLTP and CETP and age were observed. In the cell culture experiments, silencing of *ANGPTL3* in hepatocytes caused a decrease in *PLTP* expression levels. Although insignificant, a subtle decrease in PLTP activity levels was also detected in FHBL2 subjects. The data indicate that *ANGPTL3* and *PLTP* expression and secretion are linked. Intracellular PLTP has been shown to participate in lipidation (phospholipidation) of VLDL, and lower *PLTP* expression observed in *ANGPTL3*-silenced hepatocytes may reduce the rate of particle expansion of nascent VLDL (64, 75). In circulation, lower PLTP activity may contribute to decreased phospholipidation of HDL since PLTP transfers PL from VLDL/IDL to HDL during LPL-mediated lipolysis (76). Besides PLTP, endothelial lipase, a lipase that prefers hydrolysis of phospholipids, is linked to decreased HDL particle size (24). EL has been shown to be a target of ANGPTL3 inhibition (33), and therefore, increased EL activity in ANGPTL3 deficiency may be another mechanism for the lower HDL levels observed in FHBL2.

***ANGPTL3* variants**

As dyslipidemias are often very heterogeneous in nature, new candidate genes and variants linked to dyslipidemia are constantly emerging. A genetic screen of Finnish *ANGPTL3* variant carriers did not yield any obvious *ANGPTL3* loss-of-function or gain-of-function mutations in the subjects examined. Based on the screen we may conclude that out of all the variants identified, *ANGPTL3* variant rs199772471 may be a potential cause for dyslipidemia, but because only one heterozygous carrier was identified, the causality cannot be verified. In the wider candidate gene screen two homozygous carriers of rs11556624 (a variant in *STARD3*, an intracellular cholesterol-transporter protein*)* demonstrated very high TC and LDL-C, and although both subjects manifested dyslipidemia, a larger number of carriers is needed to establish causality.

7 CONCLUDING REMARKS AND FUTURE PROSPECTS

This thesis suggests a number of mechanisms related to ANGPTL3-deficiency induced FHBL2.

Our findings suggest that the FHBL2 phenotype may be a combination of liver-specific alterations in substrate utilization via GLUT2 and CD36, decreased lipidation of VLDL, combined with increased LPL activity, and decreased WAT lipolysis. ANGPTL3 protein levels are regulated by insulin, PPARγ, and LXR-mediated pathways. PLTP mass and activity may be lower in ANGPTL3 deficiency, contributing to reduced hepatic VLDL maturation and low HDL (**Figure 19**).

Based on the correlation analysis, ANGPTL3, ANGPTL4, or ANGPTL8 does not have biomarker value in predicting plasma lipid levels, and ANGPTL3 biological activity is not only regulated in a dose-dependent manner, but rather by a mechanism that may be related to its cleaving.

According to the variant data, mutations in ANGPTL3 do not play a major role in plasma TG and CE levels on a population level, as the variants with lipid-altering properties in ANGPTL3 are rare. Based on the

exome data, the association of rs1556624 and rs199772471 with dyslipidemia will need to be verified in a larger sample set.

In 2017, ANGPTL3 as a target for lipid-lowering therapy yielded positive results in clinical trials, as the monoclonal antibody against ANGPTL3 (Regeneron Pharmaceuticals) in FH patients lowered plasma TG, TC, LDL-C, and HDL significantly (77). Despite the unfavorable reduction in HDL, it seems evident that ANGPL3 deficiency is associated with protection from proatherogenesis and atherosclerotic cardiovascular disease. A future global goal will be the development of effective therapeutic antagonists of ANGPTL3 in humans (78-79).

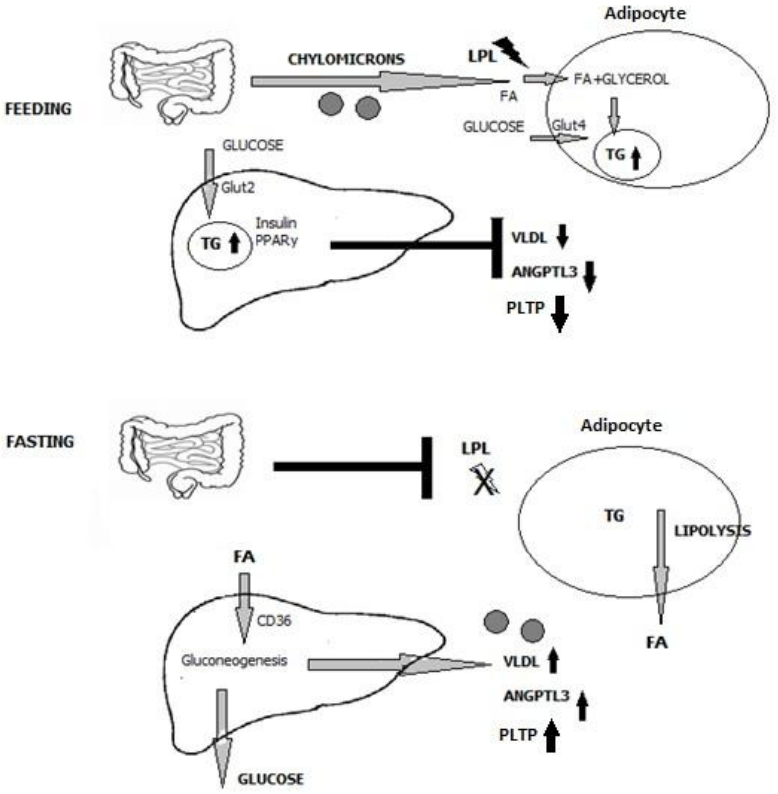

Figure 19. Summary of ANGPTL3 function. During feeding ANGPTL3 expression is down-regulated by increased insulin and PPARγ activity in the liver. WAT-LPL-mediated hydrolysis of postprandial CM-TG is increased and the resulting FFAs are rapidly taken up by CD36-mediated transport mainly into the WAT (adipocytes). Simultaneously, ANGPTL3 inhibits adipose tissue lipolysis. In the liver, increased plasma glucose concentration accelerates GLUT2-mediated glucose uptake into the tissue, decreases gluconeogenesis, decreases CD36-mediated fatty acid uptake, and decreases the secretion of TG-rich VLDL as well as the production and secretion of PLTP. During fasting the opposite effect occurs; increased ANGPTL3 inhibits LPL-WAT, which leads to activation of muscle-LPL, resulting in the hydrolyzation of VLDL-TG and the uptake of resulting FFA in the oxidative tissue. WAT lipolysis is activated and CD36-mediated uptake of FFA is increased. Hepatic gluconeogenesis is activated and glucose is secreted into the

bloodstream. Hepatic PLTP expression and secretion are increased. In ANGPTL3-deficiency, the metabolic profile is shifted towards the metabolic state observed during feeding.

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10 ORIGINAL PUBLICATIONS